

Regulatory Effect of 25-hydroxyvitamin D₃ on Nitric Oxide Production in Activated Microglia

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Microglia are activated by inflammatory and pathophysiological stimuli in neurodegenerative diseases, and activated microglia induce neuronal damage by releasing cytotoxic factors like nitric oxide (NO). Activated microglia synthesize a significant amount of vitamin D₃ in the rat brain, and vitamin D₃ has an inhibitory effect on activated microglia. To investigate the possible role of vitamin D₃ as a negative regulator of activated microglia, we examined the effect of 25-hydroxyvitamin D₃ on NO production of lipopolysaccharide (LPS)-stimulated microglia. Treatment with LPS increased the production of NO in primary cultured and BV2 microglial cells. Treatment with 25-hydroxyvitamin D₃ inhibited the generation of NO in LPS-activated primary microglia and BV2 cells. In addition to NO production, expression of 1- α -hydroxylase and the vitamin D receptor (VDR) was also upregulated in LPS-stimulated primary and BV2 microglia. When BV2 cells were transfected with 1- α -hydroxylase siRNA or VDR siRNA, the inhibitory effect of 25-hydroxyvitamin D₃ on activated BV2 cells was suppressed. 25-Hydroxyvitamin D₃ also inhibited the increased phosphorylation of p38 seen in LPS-activated BV2 cells, and this inhibition was blocked by VDR siRNA. The present study shows that 25-hydroxyvitamin D₃ inhibits NO production in LPS-activated microglia through the mediation of LPS-induced 1- α -hydroxylase. This study also shows that the inhibitory effect of 25-hydroxyvitamin D₃ on NO production might be exerted by inhibiting LPS-induced phosphorylation of p38 through the mediation of VDR signaling. These results suggest that vitamin D₃ might have an important role in the negative regulation of microglial activation.

Key Words: 25-Hydroxyvitamin D₃, 1- α -Hydroxylase, Microglia activation, Vitamin D receptor

INTRODUCTION


Microglia are macrophage-like cells in the brain and they are strongly implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease [1,2] and Parkinson's disease [3]. Microglia are activated by inflammatory and pathophysiological stimuli in neurodegenerative diseases [1], and activated microglia increase phagocytic functions by releasing cytokines, chemokine, reactive oxygen species, and other immune factors [4,5]. When microglia are activated by lipopolysaccharide (LPS), production of inducible nitric oxide synthase (iNOS) is upregulated, leading to enhanced generation of nitric oxide (NO),

which may induce neuronal damage [6-9]. Thus, understanding the mechanisms regulating microglial activation is important to reduce neuronal damage that is aggravated by activated microglia in neurodegenerative diseases.

Vitamin D₃ is a steroid hormone that is important in the regulation of plasma calcium concentration. The ultraviolet exposure of 7-dehydrocholesterol present in the skin results in the photochemical production of vitamin D₃, which is biologically inert. The liver enzyme 25-hydroxylase converts vitamin D₃ into 25-hydroxyvitamin D₃ [25(OH)D₃, cholecalciferol], the biologically active form of vitamin D₃. In the kidney proximal tubule, 1- α -hydroxylase converts 25(OH)D₃ into 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], the hormonally active vitamin D metabolite [1,10]. Immune-related cells are also reported to transform 25(OH)D₃ to 1,25(OH)₂D₃ [11-13]. Both the vitamin D receptor (1,25-dihydroxyvitamin D₃ receptor, VDR) [14-16] and 1- α -hydroxylase [15] are widely expressed in neuronal and glial cells in the brain. Although microglia express both VDR and 1- α -

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ABBREVIATIONS: LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; NO, nitric oxide; 25(OH)D₃, 25-hydroxyvitamin D₃; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; VDR, vitamin D receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SDS, sodium dodecyl sulfate; MAPK, mitogen-activated protein kinase.

hydroxylase, resting microglia produce barely detectable amounts of 1,25(OH)₂D₃ [17] while activated microglia synthesize significant amounts of 1,25(OH)₂D₃ from 25(OH)D₃ in the rat brain [17], which suggests a possible role of vitamin D₃ in microglial activation.

Some reports have suggested an effect of vitamin D₃ on neuroinflammation and microglial activation. 1,25(OH)₂D₃ inhibits the expression of iNOS in the rat brain during experimental allergic encephalomyelitis [18] and after intracranial injection of LPS [19]. It also reduces the production of proinflammatory cytokines and NO in stimulated EOC13 microglial cells [20]. Pretreatment with 1,25(OH)₂D₃ decreases 6-hydroxydopa-induced dopaminergic neuronal injury by preventing microglial activation [21]. A recent study examining the effect of dietary vitamin D on microglial activation indicated that high dietary doses of vitamin D₃ could attenuate microglia activation in a toxic model of demyelination [22]. Although most studies about the effects of vitamin D₃ were performed using 1,25(OH)₂D₃, the plasma concentration of 25(OH)D₃ is an indicator of adequate intake of dietary vitamin D₃ in many vitamin D-related clinical conditions [23,24]. Thus, to elucidate the possible role of vitamin D₃ as a negative regulator of activated microglia in neuroinflammatory conditions, the effect of 25(OH)D₃ on activated microglia should be investigated.

In this study, we have found that 25(OH)D₃ inhibited the production of NO in LPS-activated microglia through the mediation of LPS-induced 1- α -hydroxylase, and that 25(OH)D₃ exerted its inhibitory effect by preventing LPS-induced phosphorylation of p38 through the mediation of vitamin D-VDR signaling.

METHODS

Materials

LPS, *p*-nitrophenyl phosphate, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibody for β -actin was obtained from Santa Cruz Biotechnology (Paso Robles, CA, USA). Antibodies for p38, p-p38 and VDR were supplied by Cell Signaling (Danvers, MA, USA).

Culture of primary and BV2 microglial cells

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Kyung Hee University and all efforts were made to minimize the number of animals utilized as well as animal suffering. The forebrains of newborn Sprague-Dawley rats were cut into small pieces and cells were dissociated by mechanical disruption using nylon mesh. The cells were seeded into T-75 flasks. After culturing *in vitro* for 10~14 days, microglial cells were isolated from mixed glial cultures by mild trypsinization (0.05% trypsin). The purity of microglial cultures was greater than 95% as determined by isolectin B4 staining (data not shown). The immortalized murine BV2 microglial cells [25] were kindly provide by Dr. Sun-yeou Kim (Gachon University, Incheon, Korea). The BV2 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin-streptomycin.

Nitric oxide and cell viability assays

In order to determine the level of NO production, BV2 cells were plated into 96-well plates and treated with LPS (100 ng/ml) in the presence or absence of 25(OH)D₃ (1~100 nM) for 24 h. The concentration of nitrite, a soluble oxidation product of NO, in the media was determined using the Griess reaction. The supernatant (50 μ l) of each well was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was measured using a microplate reader. Sodium nitrite was used as a standard to calculate NO₂ concentration. Cell viability was measured using the MTT assay.

siRNA-mediated knockdown of gene expression

siRNA reagents against 1- α -hydroxylase and VDR were purchased from Santa Cruz Biotechnology (Paso Robles, CA, USA). BV2 cells (5 \times 10⁴) were plated in 24-well plates and transfected with 1- α -hydroxylase (20 μ mol/L) or VDR (50 μ mol/L) siRNA using DharmaFECT reagent (Dharmacon Inc, Chicago, IL, USA) according to the manufacturer's protocol. After 24 h, the culture medium was replaced with fresh supplemented medium, and the cells were cultivated for an additional 24 h.

RNA extraction and cDNA production

Total RNA was extracted using TRIzol (Gibco BRL, Gaithersburg, MD, USA) and subjected to RT-PCR using the following primers: 5'-CAGAGGCAGCCATGAGGAAC-3' (sense) and 5'-GGGTCCCTTGAAGTGGCATAG-3' (antisense) for 1- α -hydroxylase; 5'-ATGGGTCCAGAAGGACTCCTACG-3' (sense) and 5'-AGTGGTACGACCAGAGGCATAC-3' (antisense) for β -actin. The cDNAs were amplified by 28 cycles at 55°C for 45 s on a thermocycler (GeneAmp PCR System 9700, Applied Biosystems, Carlsbad, CA, USA).

Western blotting

Cells were lysed in a lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride]. Protein concentration was determined using a Bio-Rad protein assay kit. An equal amount of protein for each sample was separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk and incubated with the primary antibody (VDR, p38, p-p38, or β -actin). After removing the primary antibody, the membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody. The immune-reactive proteins were detected using an ECL reagent (Thermo Scientific, Chicago, IL, USA).

Statistical analysis

Data are presented as the mean \pm S.E.M. Student's *t*-test was used for the comparison of two independent data with normal distribution. One-way ANOVA was performed to compare multiple independent data with normal distri-

bution. p values less than 0.05 were considered significant.

RESULTS

Inhibitory effect of 25(OH)D₃ on the production of NO in LPS-activated microglia

To examine the effect of 25(OH)D₃ on the production of NO in LPS-activated microglia, we measured the amount of NO production in BV2 cells and primary cultured microglia that had been activated by LPS treatment (Fig. 1). After treatment with 100 ng/ml LPS for 24 h, the NO level in BV2 cells was 49.6 μmol/L (Fig. 1A). Production of NO in LPS-treated BV2 cells was significantly reduced in the presence of 10 and 100 μmol/L of 25(OH)D₃ (29.3 and 28.0 μmol/L of NO, respectively; Fig.1A) but was not affected in the presence of 1 μmol/L of 25(OH)D₃. In primary cultured microglia, treatment with 100 ng/ml LPS for 24 h increased the production of NO (45.2 μmol/L, Fig. 1B). Treatment with 25(OH)D₃ at a concentration of 10 and 100 μmol/L inhibited the production of NO in LPS-treated primary microglia (26.7 and 17.8 μmol/L, respectively; Fig. 1B). Cell viability was assessed using the MTT assay to rule out the possibility that the decreased NO production might be due to cell death. After BV2 cells were treated with 25(OH)D₃ at concentrations of 1, 10, and 100 μmol/L for 24 h, the cell survival rate was more than 90%. This confirms that observed effects on NO production were not caused by cell death (data not shown). These results indicate that activated microglia might be suppressed by 25(OH)D₃.

Effect of 1-α-hydroxylase siRNA on the inhibitory effect of 25(OH)D₃

An enzyme that converts 25(OH)D₃ to 1,25(OH)₂D₃, 1-α-hydroxylase, is widely distributed in microglia [15]. 1,25(OH)₂D₃ has been reported to inhibit activated microglia [18-21]. In this study, we postulated that the inhibitory effect of 25(OH)D₃ on LPS-activated microglia (Fig. 1) could have resulted either from a direct effect of 25(OH)D₃ itself

or from the effects of 1,25(OH)₂D₃ that has been converted from 25(OH)D₃ by 1-α-hydroxylase. To differentiate between these two possibilities, we examined the effect of 1-α-hydroxylase siRNA on the inhibitory action of 25(OH)D₃ in LPS-activated microglia. Fig. 2 shows that 1-α-hydroxylase is expressed in BV2 cells and primary cultured microglia and that treatment with 100 ng/ml LPS for more than 3~4 h significantly increased the expression of 1-α-hydroxylase. After the expression of 1-α-hydroxylase was blocked with 1-α-hydroxylase siRNA, the effect of 25(OH)D₃ on the LPS-activated microglia was examined (Fig. 3). In BV2 cells treated with a control siRNA, treatment with LPS

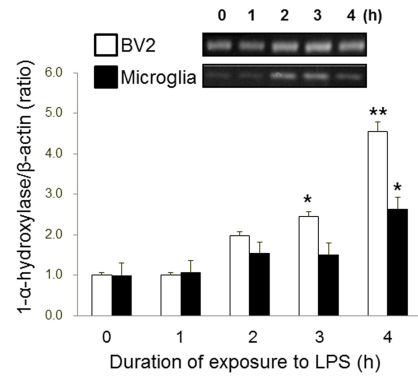


Fig. 2. Effect of LPS on the expression of 1-α-hydroxylase in BV2 cells and primary microglia. The expression of 1-α-hydroxylase mRNA began to increase at 3 h of 100 ng/ml LPS treatment in BV2 cells and at 4 h in primary microglia. The mRNA expression of 1-α-hydroxylase were normalized with β-actin. The inset shows the representative RT-PCR product of 1-α-hydroxylase mRNA in BV-2 cells and microglia. Mean values were obtained from three independent experiments. *p<0.05, **p<0.01 when compared to the untreated control group (Student's *t*-test).

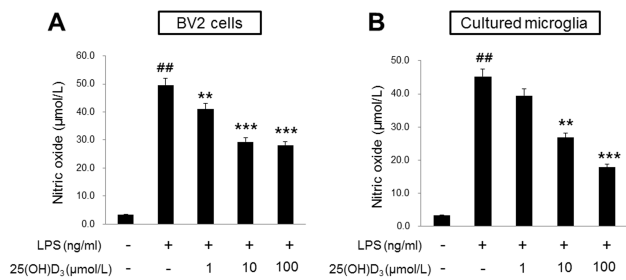


Fig. 1. Effect of 25(OH)D₃ on the production of NO in LPS-activated BV2 cells (A) and primary microglia (B). Treatment with 100 ng/ml LPS increased the production of NO in both BV2 cells and primary microglia (Student's *t*-test). 25(OH)D₃ dose-dependently (1, 10 and 100 μmol/L) inhibited the production of NO in 100 ng/ml LPS-stimulated BV2 cells and primary microglia (One-way ANOVA). Mean values were obtained from three independent experiments. **p<0.01, ***p<0.001 when compared to the LPS alone-treated group; ##p<0.001 when compared to the LPS-untreated control group.

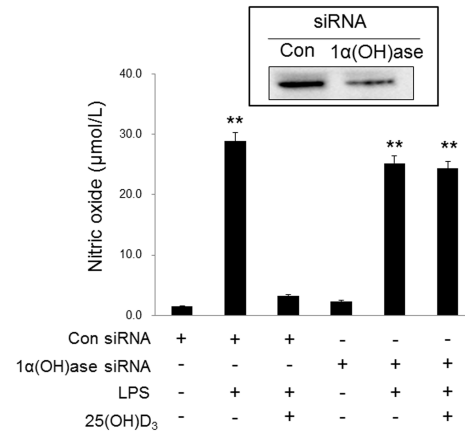


Fig. 3. Effect of 1-α-hydroxylase siRNA on the inhibitory effect of 25(OH)D₃ in LPS-activated BV2 cells. Knockdown of 1-α-hydroxylase with siRNA (20 μmol/L) suppressed the inhibitory effect of 100 μmol/L 25(OH)D₃ on the increased NO production in 100 ng/ml LPS-activated BV2 cells. The transfection efficiency of 1-α-hydroxylase siRNA was measured by Western blotting (inset). Mean values were obtained from three independent experiments. **p<0.01 when compared to the untreated control group (Student's *t*-test).

(100 ng/ml) for 36 h increased the production of NO (28.8 μ mol/L). Treatment with 100 μ mol/L 25(OH)D₃ decreased the production of NO (<10 μ mol/L) in LPS-activated BV2 cells. However, while treatment with 1- α -hydroxylase siRNA did not affect the LPS-induced increase in NO levels (25.2 μ mol/L), the inhibitory effect of 25(OH)D₃ was completely suppressed (24.3 μ mol/L of NO) in LPS-activated BV2 cells. These results indicate that LPS-induced up-regulation of 1- α -hydroxylase might be essential to the inhibitory effect of 25(OH)D₃ on LPS-activated microglia.

Effect of vitamin D receptor siRNA on the inhibitory effect of 25(OH)D₃

Glial cells, including microglia, have been reported to express VDR receptors in their nucleus [14,15]. In this study, 25(OH)D₃ inhibited LPS-activated microglia in a manner that was dependent on the hydroxylation into 1,25(OH)₂D₃ by 1- α -hydroxylase. Thus, the 1,25(OH)₂D₃ receptor VDR might be important for the inhibitory effect of 25(OH)D₃ on LPS-activated microglia. To elucidate the importance of VDR, we examined the effect of VDR knockdown on the inhibitory action of 25(OH)D₃ in LPS-activated microglia. Treatment of BV2 cells with 100 ng/ml LPS for more than 1 h significantly increased the expression of VDR (data not shown). After the expression of VDR was blocked with VDR siRNA, the effect of 25(OH)D₃ on the LPS-activated microglia was examined (Fig. 4). In BV2 cells treated with control siRNA, treatment with LPS (100 ng/ml) for 36 h increased the production of NO (35.8 μ mol/L). Treatment with 100 μ mol/L 25(OH)D₃ decreased the production of NO (9.2 μ mol/L) in LPS-activated BV2 cells. When the expression of VDR was blocked with VDR siRNA, the LPS-induced increase in the production of NO was not significantly altered (31.2 μ mol/L). However, in LPS-activated BV2 cells treated with VDR siRNA, the inhibitory effect of 25(OH)D₃ on the production of NO was completely suppressed (29.1 μ mol/L).

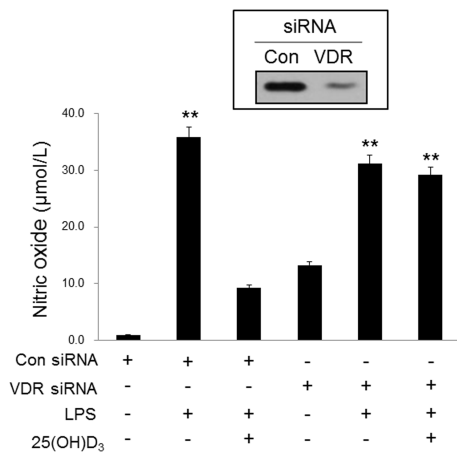


Fig. 4. Effect of VDR siRNA on the inhibitory effect of 25(OH)D₃ in LPS-activated BV2 cells. Knockdown of VDR with siRNA (50 μ mol/L) blocked the inhibitory effect of 100 μ mol/L 25(OH)D₃ on the increased NO production in 100 ng/ml LPS-activated BV2 cells. The transfection efficiency of VDR siRNA was measured by Western blotting (inset). Mean values were obtained from three independent experiments. **p<0.01 when compared to the untreated control group (Student's *t*-test).

These results indicate that 25(OH)D₃ exerts its inhibitory effect on LPS-activated microglia in a VDR-dependent manner.

Effect of 25(OH)D₃ and VDR siRNA on the p38 phosphorylation in LPS-activated microglia

It is well known that LPS-treated microglia induce inflammation through phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) [26-31]. Experiments performed in macrophages, adipocytes, prostate cells, and podocytes have shown that vitamin D exerts an anti-inflammatory effect by inhibiting p38 MAPK phosphorylation through VDR signaling [32-35]. The present study has shown that LPS-induced up-regulation of VDR is important to the inhibitory effect of 25(OH)D₃ on LPS-activated microglia. We therefore hypothesized that p38 MAPK phosphorylation might be involved in the inhibitory effect of 25(OH)D₃ on LPS-activated microglia. To elucidate this possibility, we examined the effect of 25(OH)D₃ on LPS-induced p38 phosphorylation in BV2 cells treated with VDR siRNA (Fig. 5). When BV2 cells were treated with LPS (100 ng/ml, for 36 h), the expression ratio of phosphorylated p38/total p38 was increased 1.7-fold. Treatment with 25(OH)D₃ (100 μ mol/L) inhibited the increased phosphorylation of p38 MAPK in LPS-activated BV2 cells. When BV2 cells were transfected with VDR siRNA (50 μ mol/L), the inhibitory effect of 25(OH)D₃ on the LPS-induced phosphorylation of p38 MAPK completely disappeared. These results indicate that 25(OH)D₃ reduces LPS-induced p38 phosphorylation through VDR signaling and inhibits the inflammatory response of LPS-activated microglia.

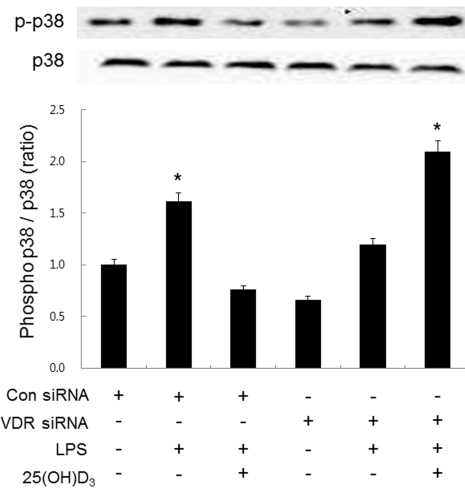


Fig. 5. Effect of 25(OH)D₃ and VDR siRNA on p38 phosphorylation in LPS-activated BV2 cells. BV-2 cells were treated with 25(OH)D₃ in the presence or absence of VDR siRNA (50 μ mol/L). Treatment with 100 μ mol/L 25(OH)D₃ inhibited the increased phosphorylation of p38 in 100 ng/ml LPS-activated BV2 cells. The extent of phosphorylation of p38 was measured by the ratio of phospho p38 to p38. VDR siRNA blocked the inhibitory effect of 25(OH)D₃ on the LPS-induced phosphorylation of p38. The inset shows the representative Western blotting product of p38 and phospho-p38 (p-p38). Mean values were obtained from three independent experiments. *p<0.05 when compared to the untreated control group (Student's *t*-test).

DISCUSSION

Microglia are activated by inflammatory stimuli in neurodegenerative diseases [1], and activated microglia increase their phagocytic function by releasing reactive oxygen species and other immune factors [4,5]. Activated microglia synthesize a significant amount of vitamin D₃ in the rat brain [17] and vitamin D₃ has an inhibitory effect on activated microglia [18-21]. Thus, it has been suggested that vitamin D₃ plays a role as a negative regulator of activated microglia. In this study, the regulatory effect of 25(OH)D₃ on LPS-stimulated microglia was investigated. We have found that the increased NO production in LPS-activated microglia is inhibited by 25(OH)D₃ in a manner that is dependent on the LPS-induced 1- α -hydroxylase. We also showed that 25(OH)D₃ exerts its inhibitory effect by inhibiting LPS-induced phosphorylation of p38 through VDR signaling.

Most studies showing the inhibitory effect of vitamin D₃ on activated microglia were performed using 1,25(OH)₂D₃ [18-21]. However, 1,25(OH)₂D₃ is formed from the hydroxylation of 25(OH)D₃ by an enzyme 1- α -hydroxylase [1,10,20,26]. Additionally, the plasma concentration of 25(OH)D₃ is an indicator of adequate intake of dietary vitamin D₃ in vitamin D-related clinical conditions [23,24]. Thus, the effect of 25(OH)D₃ on activated microglia should be investigated in order to clearly understand the mechanism of action of dietary vitamin D₃ on microglia-induced neuroinflammation in neurodegenerative diseases. In the present study, 25(OH)D₃ inhibited the NO production of LPS-activated microglia (Fig. 1). Because the 1- α -hydroxylase enzyme is widely expressed in glial cells of the brain [15], the inhibitory effect of 25(OH)D₃ could be a direct effect on activated microglia or an indirect effect through the conversion into 1,25(OH)₂D₃. In this study, LPS-stimulated microglia increased the expression of 1- α -hydroxylase (Fig. 2) and treatment with 1- α -hydroxylase siRNA completely blocked the inhibitory effect of 25(OH)D₃ on the NO production of LPS-activated microglia (Fig. 3). These results suggest that 1- α -hydroxylase in activated microglia converts 25(OH)D₃ into 1,25(OH)₂D₃, which then inhibits NO production. However, further study might be necessary to measure the production of 1,25(OH)₂D₃ in LPS-activated microglia to clearly elucidate whether 25(OH)D₃ is converted into 1,25(OH)₂D₃.

After being produced from 25(OH)D₃, 1,25(OH)₂D₃ binds VDR and initiates vitamin D-VDR signaling. Previous studies performed in multiple cell types have shown that vitamin D exerts an anti-inflammatory effect by inhibiting the phosphorylation of p38 MAPK through VDR signaling [32-35]. Activated microglia induce inflammation through the phosphorylation of p38 MAPK [26-31] and VDR is widely expressed in microglia [14,15]. Thus, it is possible that the inhibitory effect of 25(OH)D₃ on activated microglia might occur by inhibiting LPS-stimulated p38 phosphorylation through VDR signaling. In this study, the inhibitory effect of 25(OH)D₃ on activated microglia was completely blocked by VDR siRNA (Fig. 4). 25(OH)D₃ completely blocked the LPS-induced phosphorylation of p38 MAPK and this blocking effect of 25(OH)D₃ was completely abolished by VDR siRNA (Fig. 5). These results suggest that 25(OH)D₃ reduces LPS-induced p38 phosphorylation through VDR signaling, thus inhibiting the inflammatory response of LPS-activated microglia. However, it is possible that other kinases might be involved in the effect of 25(OH)D₃ on

LPS-activated microglia. Thus, p38 might be not the only kinase but one of the kinases involved in the regulatory effect of 25(OH)D₃.

It is controversial whether activation of microglia is detrimental or beneficial to neuronal cells. However, it is clear that sustained and strong inflammatory reactions mediated by microglia can trigger neuronal cell damage. Thus, it has been suggested that a negative regulatory mechanism might be present to restrict the process of microglial activation. According to the results of the present study, activated microglia might increase the expression of 1- α -hydroxylase and VDR. 25(OH)D₃ is converted into 1,25(OH)₂D₃ by 1- α -hydroxylase, which then stimulates VDR signaling and inhibits the phosphorylation of p38 in activated microglia. This cascade might inhibit the inflammatory reaction of activated microglia. In conclusion, the present study suggests that vitamin D₃ might have an important role in the negative regulation of microglial activation.

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